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Award Number: W81XWH-12-1-0009

TITLE: PKC Epsilon: A Novel Oncogenic Player in Prostate Cancer

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REPORT DATE: September 2013

TYPE OF REPORT: ANNUAL SUMMARY

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE September-2013		2. REPORT TYPE ANNUAL SUMMARY		3. DATES COVERED 1 August 2012-31 August 2013	
4. TITLE AND SUBTITLE PKC Epsilon: A Novel Oncogenic Player in Prostate Cancer				5a. CONTRACT NUMBER W81XWH-12-1-0009	
				5b. GRANT NUMBER W81XWH-12-1-0009	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Rachana Garg E-Mail: rachanag@mail.med.upenn.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pennsylvania Department of Pharmacology Perelman School of Medicine 1238 BRB II/III, 421 Curie Boulevard Philadelphia, PA 19104				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT <p>Protein kinase C epsilon (PKCϵ), a member of the PKC family of phorbol ester/diacylglycerol receptors, is up-regulated in many human cancers, including prostate cancer. We recently demonstrated that PKCϵ is an essential mediator of NF-κB activation in prostate cancer (Garg <i>et al.</i>, JBC, 287, 37570–37582, 2012). In this research, we wish to determine if PKCϵ regulates TNFα-signaling to mediate its effect on NF-κB activation. Using a specific PKCϵ antagonist, we demonstrated that PKCϵ plays essential role in the TNFα-induced phosphorylation of TNF receptor in prostate cancer cells.</p> <p>We have previously identified that PKCϵ regulates NF-κB responsive genes in prostate cancer cells, including cyclooxygenase-2 (COX-2) (JBC, 2012). COX-2 has been reported to be up-regulated in metastatic prostate cancer. As PKCϵ plays an important role in prostate cancer cell survival and cooperates with other oncogenic insults, herein we aim to determine if PKCϵ regulates COX-2 activation during prostate tumorigenesis. We observe that PKCϵ RNAi depletion diminishes constitutive and stimulated COX-2 mRNA expression and PGE2 levels in prostate cancer cells. Conversely, PKCϵ overexpression by adenoviral means potentiates COX-2 expression in LNCaP cells. Thus, our study characterizes a novel molecular link between PKCϵ and NF-κB/COX-2 and its implication in survival pathways in prostate cancer.</p>					
15. SUBJECT TERMS PKC Epsilon, Prostate Cancer, COX-2, TNF receptor					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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INTRODUCTION

The protein kinase C (PKC) isozymes represent one of the most studied Ser/Thr kinases in signaling, mitogenesis and transformation [1-2]. The PKC family has been classified into 3 classes based on their structural and biochemical properties: classical PKCs (calcium-dependent cPKCs α , β I, β II, and γ), novel PKCs (calcium-independent nPKCs δ , ϵ , η , and θ) and atypical PKCs (aPKCs ζ and ι) [3]. The first 2 groups are activated by the phorbol ester tumor promoters and the lipid second messenger diacylglycerol (DAG), a lipid generated by phospholipase C (PLC) in response to tyrosine-kinase and G-protein-coupled receptor (GPCR) activation [3]. Despite our extensive knowledge on PKC regulation and function, the specific roles for individual PKC isozymes in the context of cancer, particularly in prostate cancer, is only partially understood. Prostate cancer cells express three phorbol ester/DAG-responsive PKCs: cPKC α , and nPKCs δ and ϵ [4]. Studies from our laboratory and others have established that PKC δ generally acts as a negative regulator of proliferation and/or mediates apoptotic responses [5-6]. On the contrary, PKC ϵ is a prosurvival and mitogenic kinase [7-8]. In prostate cancer cells PKC ϵ mediates survival through Bad-dependent and Bad-independent mechanisms and is involved in the transition to androgen-independence [9-10]. PKC ϵ up-regulation is observed in >95% of human prostate specimens [11-12].

Notably, PKC ϵ emerged as a potential oncogene and is up-regulated not only in prostate cancer but also in several other epithelial cancers including lung, breast, and thyroid cancer [12-14]. Up-regulated PKC ϵ levels have also been reported in prostate tumors from TRAMP mice [12]. We recently demonstrated that transgenic overexpression of PKC ϵ in the mouse prostate causes preneoplastic lesions with elevated NF- κ B levels [15-16]. Interestingly, when we intercrossed the prostate-specific PKC ϵ transgenic mice with mice haploinsufficient for Pten, a common genetic alteration in human prostate cancer, the resulting compound mutant mice (PB-PKC ϵ ;Pten $^{+/-}$ mice) developed fully invasive adenocarcinoma. Furthermore, we demonstrated that PKC ϵ facilitates the formation of the TNFR-I complex, thereby mediating the activation of NF- κ B in prostate cancer [16]. There is ample evidence that the NF- κ B pathway is significantly dysregulated in metastatic prostate cancer and play important roles in the regulation of several cellular processes including survival, proliferation, inflammation, migration, and invasion [17-18].

The main goal of our research supported by DOD is to determine if PKC ϵ mediates the activation of COX-2, a well-known NF- κ B responsive gene, to drive essential responses relevant to prostate cancer progression. We also aim to elucidate if PKC ϵ is a mediator of TNF α signaling in prostate cancer.

BODY

1. **Role of PKC ϵ in TNF α -triggered TNF receptor phosphorylation.** The p60TNFR contains numerous potential phosphorylation sites in the cytoplasmic region [19]. We first determine if TNF α triggers phosphorylation on serine, threonine, and/or tyrosine residues of the receptor. LNCaP cells were stimulate with TNF α (25 ng/ml) for 5 min and subjected to IP with an anti-TNFR-I antibody. Western blots were probed with phosphoamino acid antibodies. It was observed that TNF α treatment mediates the phosphorylation on serine and threonine residues but not on tyrosine residues of p60TNFR (**Fig. 1A-1C**). Next, we intended to determine the involvement of PKC ϵ in TNF α -induced receptor

phosphorylation. For this set of experiment, we first inhibited PKC ϵ in LNCaP cells using the specific PKC ϵ antagonist ϵ V1-2. Cells were then stimulated with TNF α (25 ng/ml) and receptor was immunoprecipitated after 5 min. Notably, PKC ϵ inhibition significantly decreased the TNF- α -induced serine phosphorylation of the TNFR, with marginal effects on threonine phosphorylation (**Fig. 2A-B**).

2. **Mass spectrometry to identify sites in TNFR-I that undergoes phosphorylation.** To our knowledge there are no commercial phospho-TNFR antibodies available, hence we next intended to identify phosphorylation sites in TNFR-I using mass spectrometry. TNFR-I immunoprecipitated from LNCaP cells and immunoprecipitation was confirmed by Western blot. However, we could not detect a corresponding band on the Coomassie Blue gel, indicating very low yield of the immunoprecipitated TNFR-I (**Fig. 3A-B**). We then modify our approach of immunoprecipitation by covalently coupling the TNFR-I antibody onto an amine-reactive resin in the presence of sodium cyanoborohydride solution. This marginally increased the yield (**Fig. 3C-D**) as detected on a Coomassie Blue-stained gel. The TNFR-I band was excised from the gel and subjected to trypsin digestion. The peptides were enriched using immobilized metal affinity chromatography, and spotted on MALDI plate. The peptide masses obtained by MALDI analysis were used to search protein databases with search engines Mascot and Sequest. This analysis identified only 5-6 peptides corresponding to TNFR-I but none identified as a phosphoresidue bearing peptide (**Fig. 3E**). Our repeated attempts for analyzing phospho-residues by mass spectrometry were not successful because of the low yield of the immunoprecipitated TNFR-I. The possible reasons for this low yield could be the very low abundance of the endogenous receptors in cells [20]. In fact, most of the adaptor proteins that bind to the receptor were identified under the conditions of overexpression utilized in yeast two-hybrid systems or by affinity purification using recombinant proteins [20-21].
3. **Cyclooxygenase-2 (COX-2) is overexpressed in human prostate cancer cell lines akin to PKC ϵ and NF- κ B.** We first measure the protein expression of COX-2 across different prostate cell lines. As seen in **Fig. 4A**, prostate cancer cells express higher levels of COX-2 as compared to the normal prostate (RWPE-1) cells. Furthermore, this correlated with the mRNA levels as determined by qPCR (**Fig. 4B**). These results are in conjunction with the upregulated levels of PKC ϵ and NF- κ B observed in prostate cancer cell lines compared to RWPE-1 cells [16].
4. **PKC ϵ regulates LPS- or TNF α -stimulated COX-2 levels in human prostate cancer cell lines.** In the first set of experiment, LNCaP cells were transfected with either control or PKC ϵ siRNA duplexes and stimulated 48 h later with LPS (5 μ g/ml) or TNF α (20 ng/ml). RNA was extracted 4 h later. Notably, silencing PKC ϵ with two different RNAi duplexes significantly attenuated LPS- and TNF α -induced COX-2 mRNA levels (**Fig. 5**). As an alternative approach, we overexpressed PKC ϵ in LNCaP cells using an adenoviral approach. A LacZ adenovirus (AdV) was used as a negative control. Notably, ectopic expression of PKC ϵ potentiates LPS or TNF α -induced COX-2 mRNA levels (**Fig. 6**).
5. **PKC ϵ regulates constitutive COX-2 levels in human prostate cancer cell lines.** PC3 cells were transfected with either control or PKC ϵ siRNA duplexes. After 48 h, COX-2 mRNA levels were determined by qPCR. We observed that silencing PKC ϵ in PC3 cells using two different RNAi duplexes

led to a significant reduction in COX-2 mRNA levels (**Fig. 7**). Therefore, PKC ϵ mediates constitutive activation of COX-2 in androgen-independent prostate cancer cells.

6. **PKC ϵ regulates PGE2 levels in human prostate cancer cell lines.** COX is the rate limiting enzyme involved in the biosynthesis of prostaglandins and exists in two forms: COX-1 (constitutively expressed) and COX-2 (regulated isoform) [22]. Importantly, increased prostaglandins synthesis is associated with poor disease prognosis in humans [23]. PKC ϵ -depleted PC3 cells show significant decrease in PGE2 production (**Fig. 8A**). To further establish the requirement of PKC ϵ in this response, we used a pharmacological approach. The cell-permeable, Tat-fused peptide ϵ V1–2 selectively blocks the translocation and activation of PKC ϵ but not of other PKCs [16, 24]. As shown in **Fig. 8B**, ϵ V1–2 significantly inhibited PGE2 levels in PC3 cells compared with the Tat control peptide. Overall, these results strongly advocate for a role of PKC ϵ in the COX-2 and PGE2 activation in prostate cancer cells.
7. **Overexpression of PKC ϵ in “normal” prostate epithelial RWPE-1 cells increases COX-2 and PGE2 levels:** We overexpressed PKC ϵ in RWPE-1 cells, a model used as “normal” non-transformed epithelial prostate cells. PKC ϵ cDNA was subcloned into the pLenti6/V5-DEST lentiviral vector (Invitrogen). Lentiviral particles were generated using HEK293T cells and the Virapower packaging System (Invitrogen). RWPE-1 cells stably transduced with the PKC ϵ lentivirus and selected with blasticidin (0.3 μ g/ml) showed elevated PKC ϵ expression relative to the vector control (**Fig. 9A**). RWPE-PKC ϵ and parental RWPE1 cells were treated with LPS (5 μ g/ml) for 4 h and RNA was isolated. RWPE-PKC ϵ display increased COX-2 mRNA levels in response to LPS stimulation (**Fig. 9B**). Likewise, we observe significantly enhanced PGE2 levels in LPS-treated RWPE-PKC ϵ cells compared to parental cells (**Fig. 9C**).

KEY RESEARCH ACOMPLISHMENTS

- (i) We successfully demonstrated the role of PKC ϵ in TNF α -induced serine phosphorylation of TNFR-I in PC3 prostate cancer cells.
- (ii) We successfully knocked down PKC ϵ in LNCaP and PC3 cells.
- (iii) We successfully overexpressed PKC ϵ in RWPE-1 cells.
- (iv) We determined that COX-2 is overexpressed in human prostate cancer cells.
- (v) We demonstrated that PKC ϵ regulates stimulated and constitutive activation of COX-2 in prostate cancer cells.
- (vi) We demonstrated that PKC ϵ regulates PGE2 levels in prostate cancer cells.
- (vii) We found a marked induction of COX-2 and PGE2 levels in normal prostate epithelial cells upon PKC ϵ overexpression.

CONCLUSION

The main conclusions from the research carried out during the first year of DOD funding are as follows: (i) PKC ϵ mediate TNF α -induced phosphorylation of TNF receptor at serine residues. (ii) PKC ϵ mediates the activation of COX-2, a well-known NF- κ B responsive gene in prostate cancer. These findings suggest a crucial role of PKC ϵ in several important cellular processes relevant to prostate cancer progression, including survival, proliferation, metastasis and invasion. We will continue with our studies to address the role of PKC ϵ overexpression in normal prostate epithelium and how it leads to COX-2 activation. We would also put our efforts in determining if COX-2 mediates PKC ϵ responses in prostate cancer, particularly in the context of Pten loss, a common genetic alteration found in prostate cancer.

REPORTABLE OUTCOMES

Garg, R., Benedetti, L., Abera, M., Wang, H., and Kazanietz, M.G. PROTEIN KINASE C AND CANCER: WHAT WE KNOW AND WHAT WE DON'T. *Oncogene*. Accepted, In Press (2013).

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APPENDICES

FIGURE LEGENDS:

Figure 1: TNF- α treatment mediates TNFR phosphorylation on serine and threonine residues. LNCaP cells were treated with either vehicle or TNF α (25 ng/ml) and subject to IP with an anti-TNFR-I antibody. Total

and immunoprecipitated TNFR-I levels were determined by Western blot. IgG was used as a control for the immunoprecipitation. TNFR-I immunoprecipitates were analyzed for phosphorylation by Western blot using (A) anti-phosphotyrosine (PY20), (B) anti-phosphoserine, or (C) anti-phosphothreonine antibodies.

Figure 2: Effect of PKC ϵ on TNF α -triggered TNFR phosphorylation. LNCaP cells were treated with the PKC ϵ specific inhibitor ϵ V1-2 or control Tat carrier peptide (1 μ M, 1 h) and then stimulated with TNF α (25 ng/ml) or vehicle. After 5 min of TNF α stimulation, TNFR-I was immunoprecipitated with an anti-TNFR-I antibody. The effect of PKC ϵ inhibition on receptor phosphorylation was determined by Western blot in TNFR-I immunoprecipitates using (A) anti-phosphoserine and (B) anti-phosphothreonine antibodies.

Figure 3: Mass spectrometry to identify phosphorylation sites in TNFR-I. LNCaP cells (10×10^6) were treated with TNF α (25 ng/ml) or vehicle for 5 min. Cleared cell lysates were immunoprecipitated with an anti-TNFR-I antibody, washed, and resolved by SDS/PAGE. IgG was used as a control. (A) Western Blot to confirm TNFR-I immunoprecipitation. (B) Coomassie blue stained gel picture. Molecular weight markers in kDa are indicated. TNF α -stimulated LNCaP cell lysates were immunoprecipitated using a TNFR-I antibody that is coupled to an amine-reactive resin in the presence of sodium cyanoborohydride solution. (C) Western blot to confirm the TNFR-I immunoprecipitation. (D) Coomassie blue stained gel picture. The arrow indicates the position of the band that was excised for enzymatic digestion by trypsin and subsequent mass spectrometric analysis. (E) List of some of the molecules identified from the in-gel digest. Number of tryptic peptides observed by MALDI analysis that matched tryptic peptides derived from a given protein in the database is shown. Arrow indicates the identification of TNFR-I. List has been curtailed from a total of 66 proteins identified.

Figure 4: COX-2 is overexpressed in human prostate cancer cell lines. (A) Protein and (B) mRNA expression of COX-2 in different prostate cell lines. *, $p < 0.05$ vs. RWPE-1 cells ($n=3$).

Figure 5: Silencing PKC ϵ reduces LPS- or TNF α -stimulated COX-2 levels in human prostate cancer cell lines. LNCaP cells were transfected with two different PKC ϵ RNAi duplexes (ϵ 1 or ϵ 2) or a non-target control RNAi duplex (*NTC*). After 48 h, cells were stimulated with LPS (5 μ g/ml) or TNF α (20 ng/ml) or vehicle control for 4 h and RNA was isolated. PKC ϵ depletion was confirmed by qPCR. Results were normalized to endogenous 18 S. *, $p < 0.05$ vs. control ($n=3$).

Figure 6: Overexpression of PKC ϵ potentiates LPS- or TNF α -stimulated COX-2 levels in human prostate cancer cell lines. LNCaP cells were infected with PKC ϵ AdV or LacZ control (10 MOI). After 48 h, cells were stimulated with LPS (5 μ g/ml) or TNF α (20 ng/ml) or vehicle control for 4 h and RNA was isolated. PKC ϵ overexpression was confirmed by qPCR. Results were normalized to endogenous 18 S. *, $p < 0.05$ vs. control ($n=3$).

Figure 7: PKC ϵ regulates constitutive COX-2 levels in human prostate cancer cell lines. PC3 cells were transfected with either control or PKC ϵ siRNA duplexes. After 48 h, COX-2 mRNA levels were determined by qPCR. Results were normalized to endogenous 18 S. *, $p < 0.05$ vs. control ($n=3$).

Figure 8: PKC ϵ regulates PGE2 levels in human prostate cancer cell lines. PC3 cells were transfected with either control or PKC ϵ siRNA duplexes. After 48 h, PGE2 levels (normalized to total protein), were measured in the culture media using an EIA kit. *, $p < 0.05$ vs. control (n=3).

Figure 9: Overexpression of PKC ϵ in “normal” prostate epithelial RWPE-1 cells increases COX-2 and PGE2 levels. (A) RWPE-1 cells were infected with ViraPower lentiviruses containing a human PKC ϵ construct cloned into pLenti6/V-5 DESTRWPE-1. Expression of PKC ϵ was analyzed by Western blot using an anti-PKC ϵ antibody. Vinculin was used as control. RWPE-1 cells overexpressing or parental cells were stimulated with LPS (5 μ g/ml) or vehicle control for 4 h and RNA was isolated. (B) COX-2 mRNA levels were determined by qPCR. (C) PGE2 levels (normalized to total protein), were measured in the culture media using an EIA kit. *, $p < 0.05$ vs. control (n=3).

Figure 1

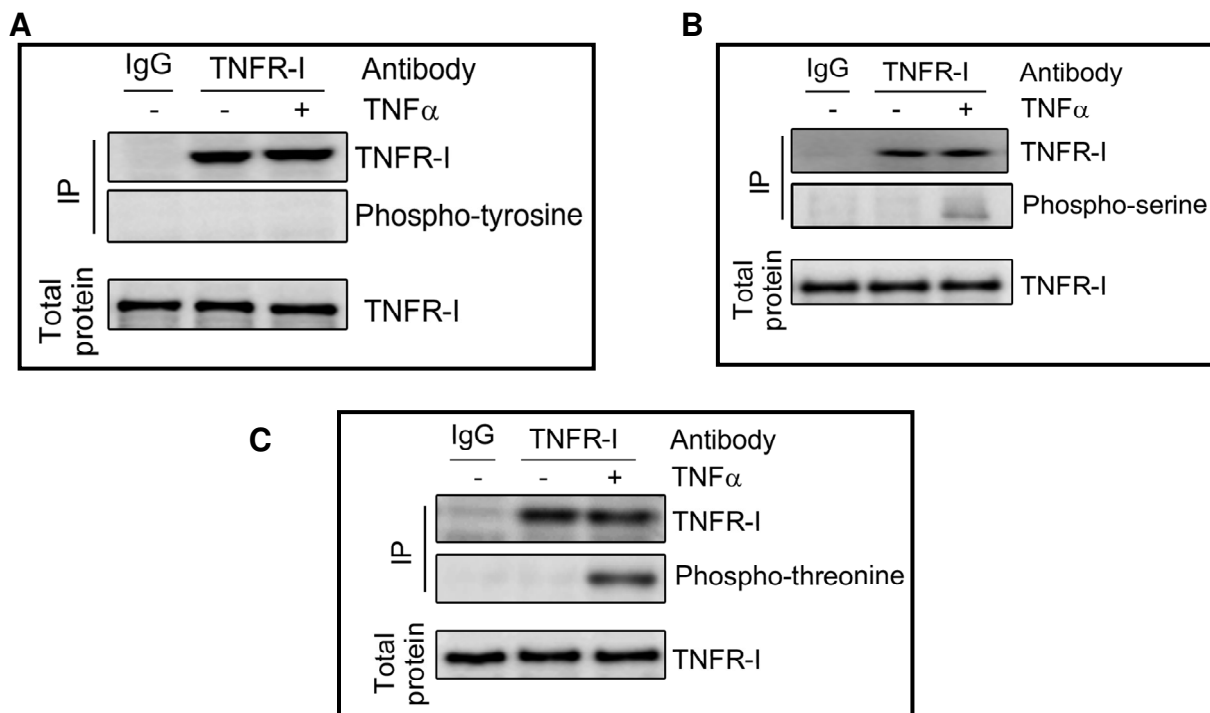


Figure 2

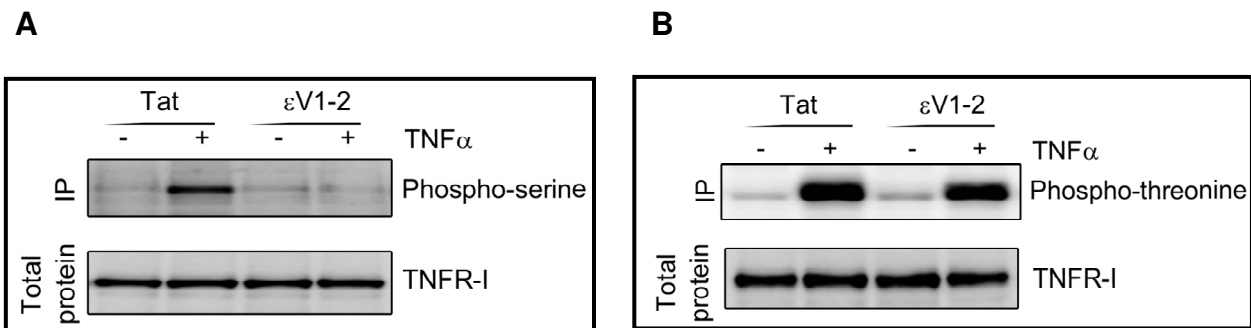
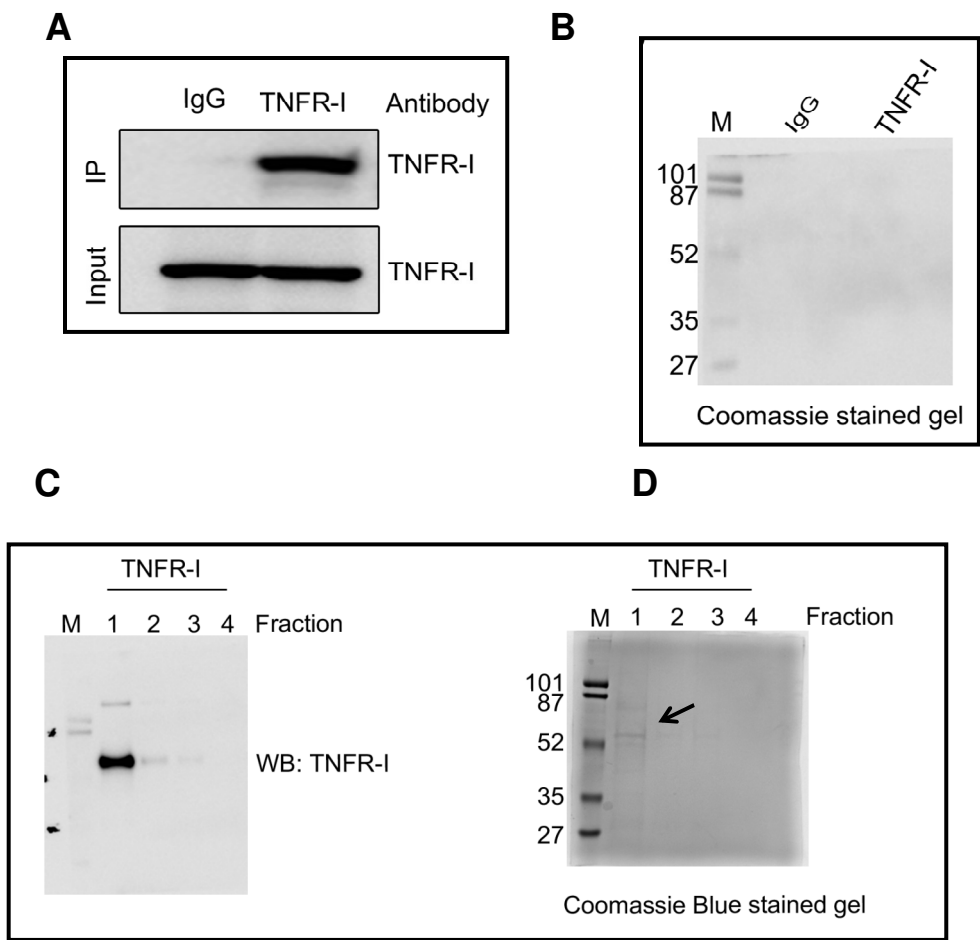


Figure 3



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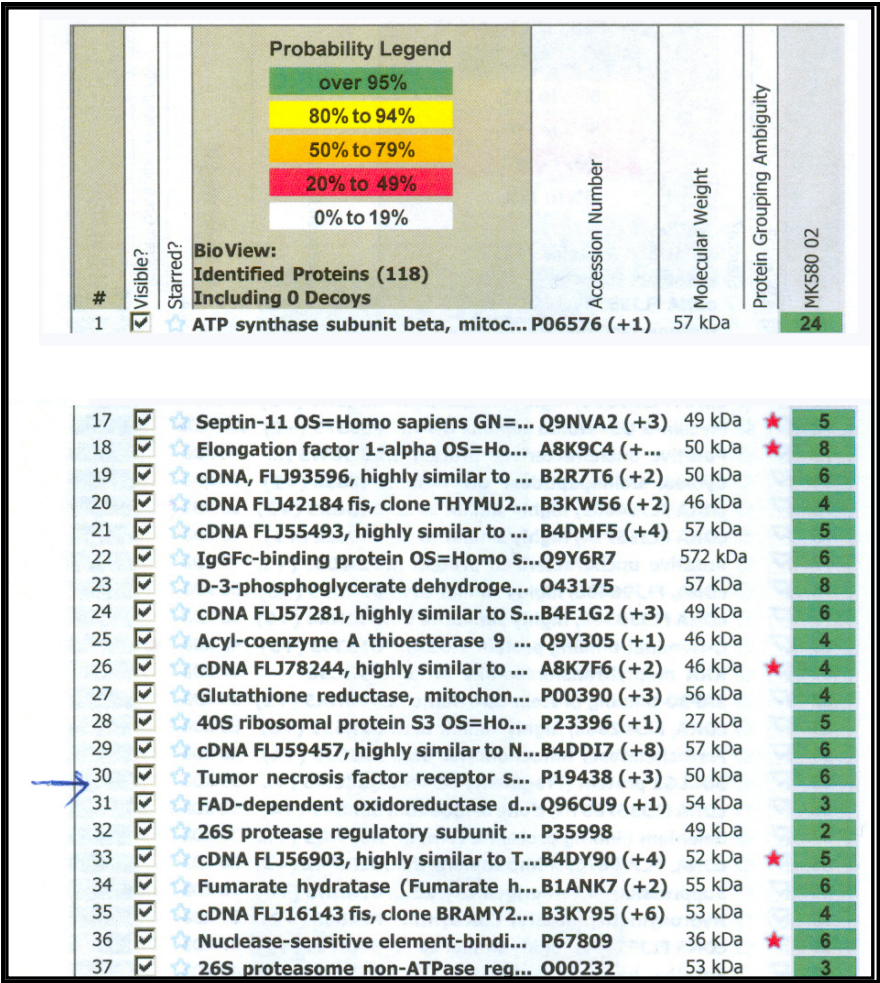


Figure 4

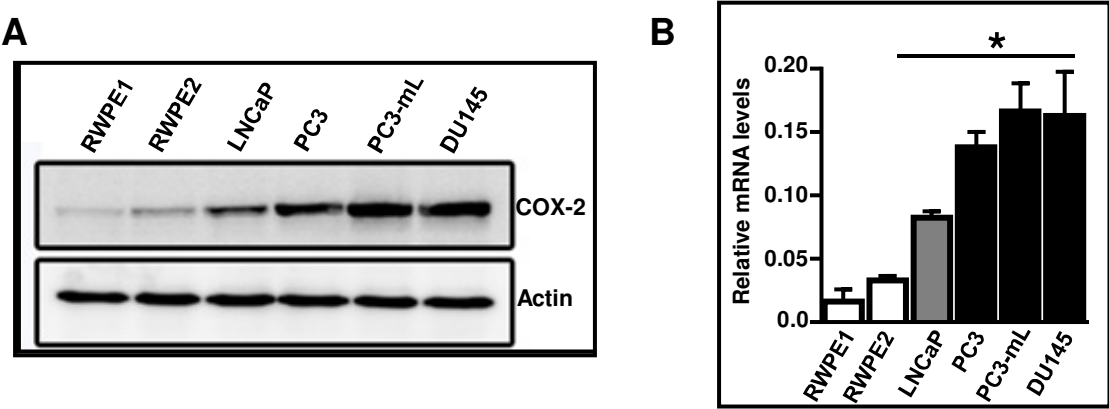


Figure 5

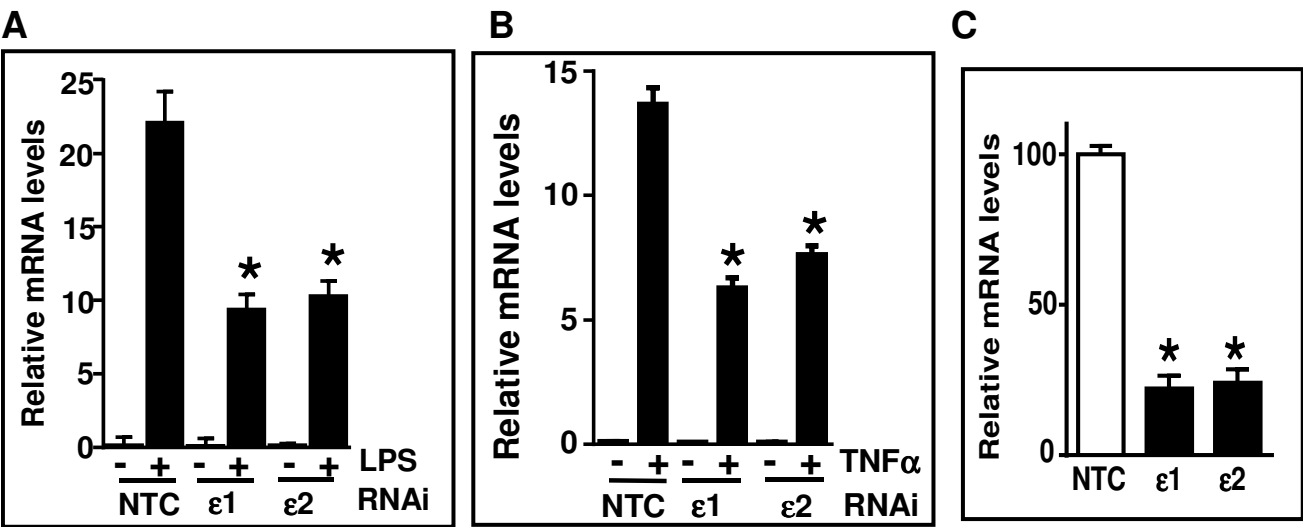


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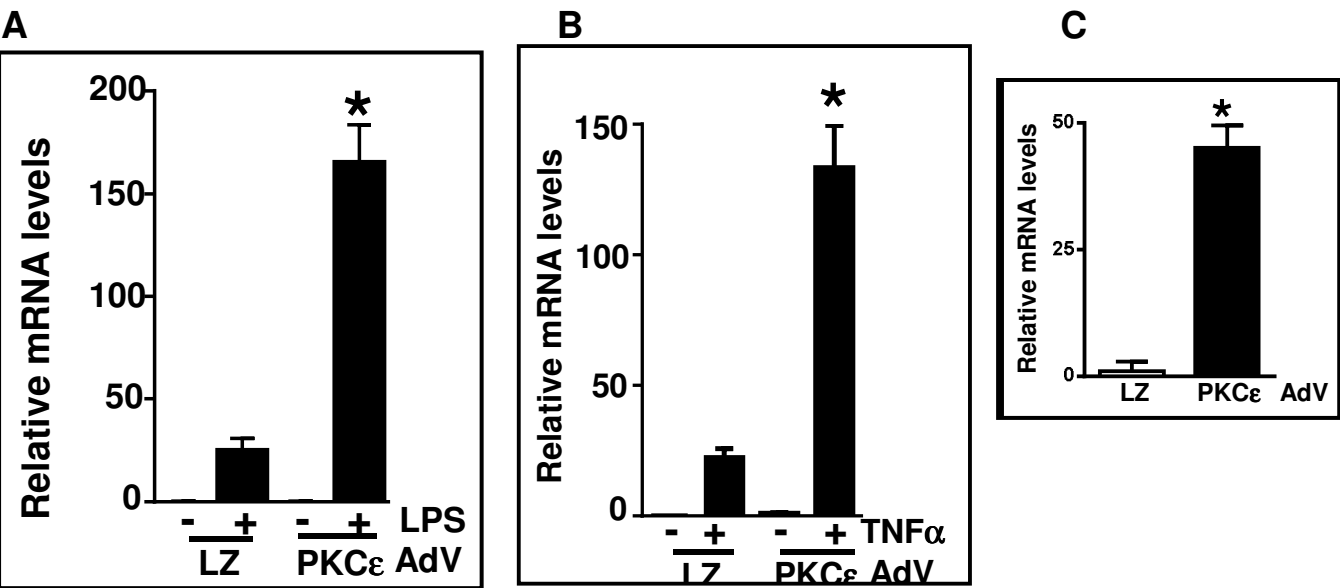


Figure 7

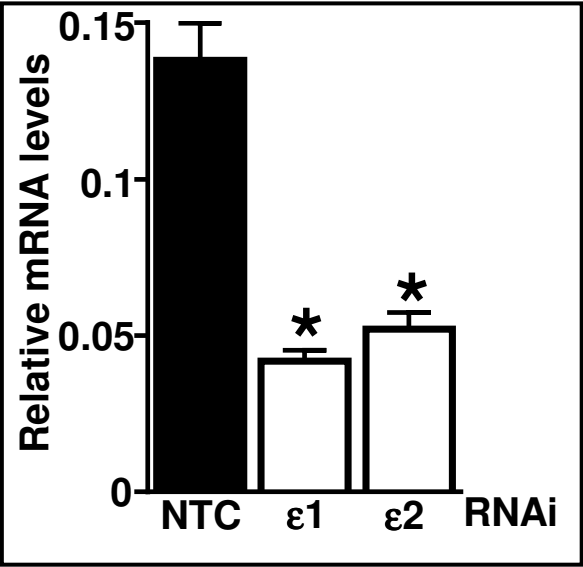
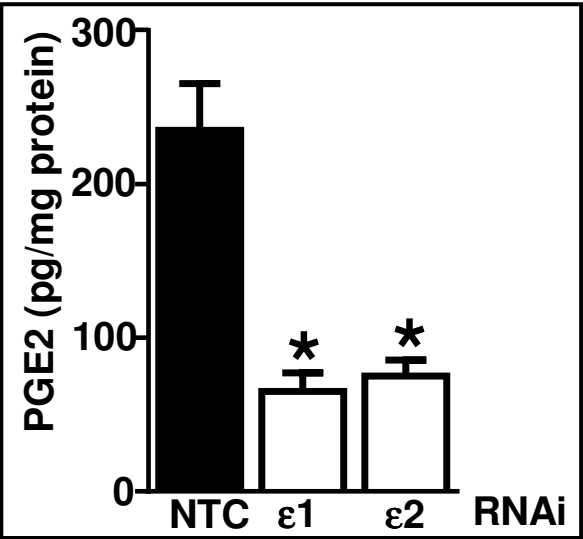


Figure 8

A



B

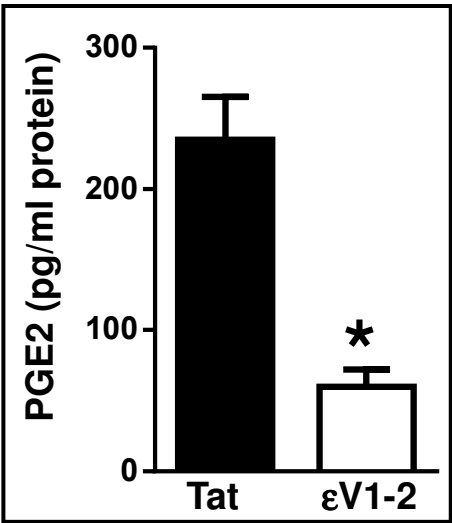
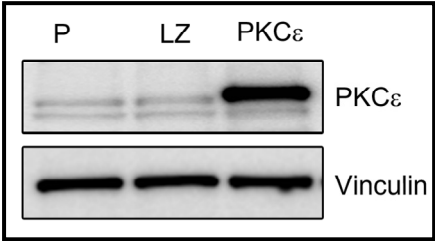
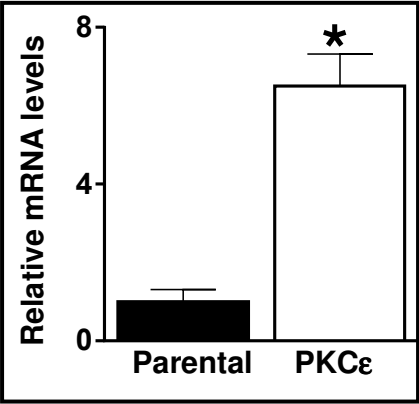


Figure 9

A



B



C

